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(54) Title: MYOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

(57) Abstract

Compositions of matter which comprise a mixture of isolated muscle precursor cells with either isolated mesenchymal stem cells or isolated periosteum cells, optionally further including a myoinductive agent, a method for inducing isolated human mesenchymal stem cells to differentiate into myogenic cells, a method for producing dystrophin-positive myogenic cells in a mammal, a method for effecting muscle regeneration, and a method for treating muscular dystrophy are disclosed.

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MYOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

This invention relates to the field of muscle regeneration and, more particularly, to the production of dystrophin-positive cells in dystrophin-negative subjects.

A mesenchymal stem cell (MSC) is a pluripotential progenitor cell that has the capacity to divide many times and whose progeny eventually gives rise to mesodermal tissue such as: cartilage, bone, muscle, fat and tendon. A population of these pluripotential stem cells has been shown to be present in embryonic limb buds of chicken, mouse and human (2, 5, 13, 26, 27). Such stem cells have also been shown to exist in postnatal and adult organisms. Friedenstein, Owen (8, 22) and others reported that cells derived from bone marrow have the capability to differentiate into osteogenic cells when assayed in diffusion chambers. It has been documented that such cells when loaded in ceramic cubes and implanted to subcutaneous or intramuscular sites, possess the ability to form bone and cartilage tissue (10, 21). Furthermore, the periosteum has been reported to contain MSCs with the same ability to form bone or cartilage in ceramic cubes or diffusion chambers (20).

With regard to myogenic potential of MSCs, rat and mouse clonal embryonic cell lines have been shown to transform into myoblasts and form myotubes after exposure to 5-azacytidine or 5-azadeoxy cytidine. This work is disclosed in copending, commonly assigned U.S. application serial no. 08/377,461, filed January 24, 1995 and entitled "Lineage-Directed Induction of Human Mesenchymal Stem Cell Differentiation." The same cells also exhibit adipocytic and chondrogenic phenotypes (11, 17, 21, 37). However, the expression of myogenic properties of stem cells harvested from postnatal sources has not been observed in humans.

The *mdx* strain of mice is well recognized and has been utilized as an animal model correlated with Duchenne-type human muscular dystrophy (5). Although *mdx* mice do not exhibit severe clinical features of myopathy, their skeletal and cardiac muscles are composed of predominantly dystrophin-negative myofibers and show an extensive myopathic lesion accompanied with muscle fiber necrosis and degeneration (10). Injection of myoblasts (satellite cell-derived), also referred to herein as muscle precursor cells(MPCs), has been observed to result in the conversion of dystrophin-negative myofibers to dystrophin-positive ones (25, 30), giving hope that this procedure may provide a useful treatment for the patients affected with inherited myopathies (18, 24, 26). One major drawback in this myoblast transfer therapy is that a large number of myoblasts are needed to bring about a clinically relevant effect.

The present invention is based on the discovery by the inventors that suitable environmental conditions induce human mesenchymal stem cells (hMSCs) to express a myogenic phenotype. Such conditions can be established in developing myogenic cell culture or in regenerating and

reforming dystrophin-positive muscles. The procedures described here elucidate the behavior of marrow- or periosteal- derived hMSCs in the presence of muscle precursor cells (MPCs) *in vitro* and *in vivo*.

Because, *inter alia*, hMSCs can be mitotically expanded in culture and their harvest is less destructive than muscle biopsies used to harvest myoblasts, these cells have been discovered by the inventors to have significant advantages over the use of myoblasts in treating myopathies. Additionally, there are several sources of hMSCs in the human body, and their harvest is less destructive than muscle biopsies used to harvest MPCs.

With the above in mind, the inventors studied the behavior of hMSCs co-cultured with myoblasts, and also injected syngeneic normal MSCs into muscles of the mdx mouse to evaluate their capability to produce normal muscle fibers.

Accordingly, in one aspect the invention provides a composition of matter which comprises a mixture of isolated muscle precursor cells and isolated human mesenchymal stem cells. Preferably, the mesenchymal stem cells are marrow-derived or periosteum-derived. Also, the composition is preferably a composition of entirely human cells. The composition can further include a myoinductive agent, such as 5-azacytidine or 5-azadeoxycytidine.

Another aspect of the invention provides a composition of matter which comprises a mixture of isolated muscle precursor cells and isolated periosteum cells. Here also, the composition is preferably a composition of entirely human cells. This composition also can further include a myoinductive agent, such as 5-azacytidine or 5-azadeoxycytidine.

Another aspect of the invention provides a method for inducing isolated human mesenchymal stem cells to differentiate into myogenic cells by maintaining the cells in the presence of muscle precursor cells. The isolated human mesenchymal stem cells can be maintained in the presence of muscle precursor cells *in vitro* or *in vivo*.

Another aspect of the invention provides a method for producing dystrophin-positive myogenic cells in an individual by administering to the individual a myogenic cell producing amount of isolated human mesenchymal stem cells or of any of the compositions of the invention.

Another aspect of the invention provides a method for effecting muscle regeneration in an individual in need thereof by administering to an individual in need of muscle regeneration a muscle regenerative amount of isolated human mesenchymal stem cells or of any of the compositions of the invention.

Another aspect of the invention provides a method for treating muscular dystrophy in an individual so afflicted by administering to that individual an amount of isolated human mesenchymal stem cells or of any of the compositions of the invention effective to produce dystrophin-positive myogenic cells in said individual.

The following is a brief description of the drawings which are presented only for the purposes of further illustrating the invention and not for the purposes of limiting the same.

Figures 1A-1C are photomicrographs showing the histology of the right tibialis anterior muscle of mdx mouse at 6 weeks after injection (A). There is a large

area of a typical myopathic lesion accompanied with cellular infiltration. Inside of the lesion, swollen and vacuolated myotubes are found. At 8 weeks post-injection (B), the regenerating area contains clustered muscle fibers of small diameter, surrounded by large closely packed normal muscle fibers. At a higher magnification (C), a typical regenerating fibers are shown having large centrally located nuclei. Bar = 100 μm.

Figures 2A-2C are photomicrographs showing dystrophin expression in the muscle of normal mouse (A). The entire circumference of muscle fibers are clearly stained with anti-dystrophin antibody. Anti-dystrophin antibody localization in the muscle of mdx mouse 8 weeks after injection of myoblasts (B). Immunostaining clearly demonstrates multiple dystrophin-positive fibers surrounded by dystrophin-negative myotubes. The shape and size of the positive fibers are more varied than those of a normal muscle. There are few dystrophin-negative fibers intermingled with the positive fibers (*). Dystrophin expression in the muscle of an *mdx* mouse 10 weeks post-injection with MSCs (C). The intensity of staining is almost same as that in the muscle injected with myoblasts. There are contiguous dystrophin-positive muscle fibers, forming a cluster.

Skeletal muscles develop in a specific sequence of cellular differentiation, which includes: the commitment of progenitor cells into myoblasts, the proliferation of myoblasts, their fusion to form multinucleated myotubes and the sequential expression of muscle specific proteins. Each of these steps is controlled by a complex spectrum of intrinsic and extrinsic biological factors (38). Particularly, in the process of myoblast fusion, there is a clear molecular recognition mechanism which allows

myoblasts to fuse only to cells of their own lineage, prohibiting their fusion to other cell types such as osteogenic, chondrogenic and fibroblastic cells (38,40).

Mesenchymal stem cells are the formative pluripotential blast cells found *inter alia* in bone marrow, blood, dermis and periosteum that are capable of differentiating into any of the specific types of mesenchymal or connective tissues (*i.e.* the tissues of the body that support the specialized elements; particularly adipose, osseous, cartilaginous, elastic, and fibrous connective tissues) depending upon various influences from bioactive factors, such as cytokines. Although these cells are normally present at very low frequencies in bone marrow, a process for isolating, purifying, and greatly replicating these cells in culture, *i.e. in vitro,* is disclosed in PCT Published Application No. WO 92/22584 (published 23 December 1992).

Homogeneous human mesenchymal stem cell compositions are provided which serve as the progenitors for all mesenchymal cell lineages. MSCs are identified by specific cell surface markers which are identified with unique monoclonal antibodies. The homogeneous MSC compositions are obtained by positive selection of adherent marrow or periosteal cells which are free of markers associated with either hematopoietic cell or differentiated mesenchymal cells. These isolated mesenchymal cell populations display epitopic characteristics associated with only mesenchymal stem cells, have the ability to regenerate in culture without differentiating, and have the ability to differentiate into specific mesenchymal lineages when either induced *in vitro* or placed *in vivo* at the site of damaged tissue.

In order to obtain human mesenchymal stem cells, it is necessary to isolate rare pluripotent mesenchymal stem cells from other cells in the bone marrow or other hMSC source. Bone marrow cells may be obtained from iliac crest, femora, tibiae, spine, rib or other medullary spaces. Other sources of human mesenchymal stem cells include embryonic yolk sac, placenta, umbilical cord, fetal and adolescent skin, and blood.

The method of their isolation comprises the steps of providing a tissue specimen containing mesenchymal stem cells, adding cells from the tissue specimen to a medium which contains factors that stimulate mesenchymal stem cell growth without differentiation and allows, when cultured, for the selective adherence of only the mesenchymal stem cells to a substrate surface, culturing the specimen-medium mixture, and removing the non-adherent matter from the substrate surface.

Compositions having greater than 95%, usually greater than 98% of human mesenchymal stem cells can be achieved using the previously described technique for isolation, purification and culture expansion of MSCs. The desired cells in such compositions are identified as $SH2^+$, $SH3^+$, $SH4^+$ and CD^- and are able to provide for both self renewal and differentiation into the various mesenchymal lineages. Ultimately, repair and regeneration of various mesenchymal tissue defects could be accomplished starting from a single mesenchymal stem cell.

Example 1

Dystrophin Expression in *mdx* Mice Using hMSCs

Mdx mice have been utilized as an animal model of Duchenne-type human muscular dystrophy. Although *mdx* mice

do not exhibit severe clinical features of myopathy, their skeletal and cardiac muscles are composed of predominantly dystrophin-negative myofibers and show an extensive myopathic lesion accompanied with muscle fiber necrosis and degeneration. Injection of myoblasts (satellite cell-derived) has been observed to result in the conversion of dystrophin-negative myofibers to dystrophin-positive ones, which gives hope that this procedure may provide a useful treatment for patients affected with inherited myopathies. Therefore, the *mdx* mouse was adopted as an experimental animal model in these studies. One major drawback in this myoblast transfer therapy is that a large number of myoblasts are needed to bring about a clinically relevant effect. Because MSCs can be mitotically expanded in culture and their harvest is less destructive than muscle biopsies used to harvest myoblasts, these cells may be an alternative source of cells for treating myopathies. With the above in mind, we have studied the behavior of MSCs co-cultured with myoblasts or injected into either syngeneic normal muscle or into the *tibialis anterior* muscle of *mdx* mice, in order to evaluate the capability of MSCs to produce normal muscle fibers.

MATERIALS AND METHODS

Bone Marrow Cell Preparation

As sources of bone marrow MSCs, femora of male mice (C57B1/10 SNJ, Jackson Laboratories, Bar Harbor, ME) of 6 to 10 weeks of age were used. After the soft tissue was completely removed to avoid contamination by myogenic precursors, the distal and proximal ends of the femora were cut with a rongeur and the marrow plugs were removed from the shafts of the bones by expulsion with a syringe filled with complete medium and fitted with, for rats, an 18-gauge needle and, for mice, a 20-gauge needle. Complete medium

consisted of BGJ_b (Fitton-Jackson Modification; Sigma; St. Louis, MO) with antibiotics (100 μ/ml sodium penicillin G, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B: Gibco BRL, Grand Island, NY), and 10% fetal calf serum (selected batches; JRH Biosciences, Lenexa, KS). Marrow cells were dispersed several times through 20- and 22-gauge needles. The cells were gently centrifuged, resuspended in complete medium, counted and plated at a density of 5.0×10^7 cells/100-mm dish. At 3 days of culture, the medium was removed, the dishes were washed twice with Tyrode's salt solution (Sigma, St. Louis, MO) and fresh complete medium added; the medium was changed twice a week.

Preparation of Mouse Myoblasts

Muscle tissue was obtained from the *tibialis anterior* and *quadracepts* muscles of three-week-old mice. The dissected muscle was digested with 0.2% trypsin for 35 min and the reaction terminated by the addition of fetal bovine serum at one half the sample volume. The cell suspension was centrifuged for 5 min at 300 xg, resuspended in 10 ml of DMEM-LG containing 20% fetal calf serum, triturated at least 20 times with a 10-ml disposable pipette, and then passed through a 100-μm Nitex filter (to remove cell aggregates and debris). Prior to seeding, the cells were preplated into a 100-mm culture dish and incubated for 30 min to reduce the number of fibroblasts. The nonadherent muscle precursor cells were counted in a hemocytometer and the cell density was adjusted to 1.0×10^5 cells/ml with DMEM-LG supplemented with 20% fetal calf serum. Cells were seeded onto 35 mm plates at 1.0×10^5 cells per plate, in 1 ml, and the medium changed every 2 days. At the onset of cell fusion (4-5 days), the medium was changed to DMEM-LG with 5% fetal calf serum. The cultures were maintained until the overt formation of myotubes has ceased; usually by 11 days following seeding.

In Vivo Injections

Breeding pairs of both *mdx* mice (C57b1/10mdx) and normal mice (C57b1/10snj) were purchased from Jackson Laboratories. Newborn *mdx* mice were raised for 3 weeks, separated from their parents and immediately used as injection hosts. Normal newborn mice were sacrificed within 3 days of birth for collection of syngeneic myoblasts. Normal adult male mice were utilized as a source of bone marrow-derived MSCs.

Myoblasts were obtained from normal syngeneic newborn muscle tissue as described above, then centrifuged, washed with Tyrode's salt solution and counted. MSCs were released with trypsin-EDTA after 10 to 14 days of primary culture, washed with Tyrode's salt solution and counted. The densities of both cell suspensions were adjusted with serum-free BGJ_b to 5.0×10^5 cells/5 μl.

Mdx mice were anesthetized by limited exposure to CO_2 gas. The hindlimbs were sterilized with 70% ethanol, and a longitudinal skin incision was made along the anterior edge of the tibia with a No. 11 scalpel. The *tibialis anterior* muscle was identified and a needle connected to a microliter syringe (Hamilton Company, Reno, NV) containing 5 μl of cells was inserted into the interior of the muscle at a low angle, and the full 5 μl of cells were injected into the middle of the muscle. The right *tibialis anterior* was injected with cells and the left side injected with BGJ_b medium only. The skin was sutured in an ordinary manner and closed.

Tissue Section Immunohistochemistry

At 6, 8 and 10 weeks after injection, mice were sacrificed, and the muscle mass located on the anterior surface of the tibia was excised. The mass of each muscle

sample was measured prior to cutting the muscle in half. The proximal half was immersed in OCT compound (Tissue-Tek, Miles Laboratories, Inc., Napperville, IL) and then snap-frozen in liquid nitrogen-cooled in 2-methylbutane for cryosectioning and immunohistochemical staining; the distal half was fixed with 10% neutral formalin for routine histology.

The frozen samples were sectioned with a cryostat, and 8 μm sections were placed on glass slides coated with poly-L-lysine. Prior to staining, sections were blocked in phosphate-buffered saline containing 20% horse serum (JRH Biosciences, Lenexa, KS; PBS+HS, pH 7.4). The sections were immunostained with anti-dystrophin antibody as described above. Following incubation in the first and second antibodies, they were washed and incubated for 15 min with Texas red conjugated avidin (Organon Teknika Corp., West Chester, PA) diluted 1 to 6000 with PBS+HS and washed 4 times in PBS+HS for 5 min. The slides were mounted in glycerol: PBS (9:1), pH 8.5 containing 0.01 M p-phenylenediamine (PPD) (Eastman Kodak, Rochester, NY) and observed with a fluorescent microscope (BH-2, Olympus, Tokyo).

Histologic Evaluation

For analysis of the dystrophin content in the transverse section of the muscle, the section was subdivided into 6 areas and a micrograph of the region containing the largest number of dystrophin-positive myofibers in each area was recorded with Kodak TMAX 400 film. From full frame prints, the number of dystrophin-positive and -negative myofibers was tabulated and the percentage of positive to total number of myofibers in the 6 areas was calculated. The average values of the right muscle (cell-injected) were compared with that of the left

(cell medium-injected). Sections of the other half of the muscle samples were stained with hematoxylin and eason for histologic evaluation.

RESULTS

In vivo Muscle Injections

A total of 52 *mdx* mice were used for the injections in this study. Muscles were harvested at 6, 8 and 10 weeks after injection; 29 mice were injected with MSCs and 23 with myoblasts. During harvest, muscles were dissected *in toto* and immediately weighed. Table II contains the mass ratios between right and left muscles harvested at 6, 8 and 10 weeks after injection. The data were then analyzed both in total and according to sex; this data is summarized in Table 1.

Table 1

Summary of 6, 8 and 10 Week Post-Injection Muscle Mass

mg muscle (standard deviation)

	Total Female	Total Male	Total F + M
Myoblast-injected			
Cell-injected	93.2 (8.5)†	100.6 (10.1)§	97.5 (10.0)¶
Medium-injected	85.7 (12.6)	88.9 (26.0)	87.6 (21.1)
Percent increase	8.8	13.1	13
Number	10	13	23
Mesenchymal Stem cell-injected			
Cell-injected	78.2 (12.2)¶	97.9 (12.2)	85.6 (15.9)[
Medium-injected	74.7 (10.5)	96.8 (9.5)	83.1 (14.7)
Percent increase	5.4	0.9	3.0
Number	18	11	29

All cell-injected muscle groups showed statistically significant increases in muscle mass compared to contralateral controls except for the male MSC-injected group. There was an increase in muscle mass of this group but not statistically significant (p value = 0.7). The myoblast-injected muscle showed nearly a 4-times greater percent change in muscle mass (11.1%) than that of MSC-injected muscle (3.0%).

The histology of the muscle samples showed various degrees of degeneration and/or regeneration in both MSC- and periosteal cell-injected groups. At 6 weeks after injection, large foci of cellular infiltration were found (Fig. 1A). However, at 8 to 10 weeks, these areas were reduced in size and were surrounded by myotubes (Fig. 1B).

The transverse sections appeared normal, except that the nuclei were centrally located as compared to the normal peripheral location (Fig. 1C). The frequency of small, basophilic, centrally nucleated and vacuolated myotubes increased in number with time. Although there were areas of fat cell accumulation in some few samples, calcified myofibers and massive fibrosis were not observed. There was no evidence of bacterial infection in any of the muscle specimens.

Immunohistochemical staining for dystrophin served as a marker for normal muscle differentiation. In the normal mouse (*snj*), dystrophin is localized to the entire circumference of the plasmalemma on every myotube (Fig. 2A). IN the myoblast-injected group, a wide variation of the extent of dystrophin-positive myofibers was observed. The shape and diameter of teh positive fibers were more irregular than those of normal syngeneic mice (Fig. 2B). Of interest was the finding that some of the fibers with the centrally located nuclei were also dystrophin-positive.

Quantitation of immunoreactivity for samples harvested at 6, 8, and 10 weeks after injection showed a consistent pattern for the percentage of dystrophin-positive fibers within each experimental group (Table 2).

Table 2

Incidence of Dystrophin-Positive Muscle Fibers

	Proportion of Positive Fibers (%)		
	6 wk	8 wk	10 wk
Injected			
MSC (R)	2.4±1.7 (9)	2.6±1.6 (9)	5.7±9.4 (11)
Sham (L)	0.3±0.5 (9)	0.5±0.6 (9)	0.3±0.5 (11)
MPC (R)	15.4±19.1 (9)	35.8±16.7 (7)	26.6±23.2 (8)
Sham (L)	0.8±0.8 (8)	0.3±0.6 (7)	3.0±2.0 (8)

At each harvest time, the myoblast-injected samples had, by far, the largest regions of dytrophin-positive myotubes, followed by the MSC-injected group, and then by the medium-injected control group. The percentage of positive myofibers for the myoblast-injected group was from 5 to 10 times greater than that for the MSC-injected group. However, the MSC-injected group had 8 to 19 times more dystrophin-positive myofibers than the medium-injected muscles. There was a significant difference in the average values between the MSC-injected and medium-injected muscles at 6 and 8 weeks ($P<0.05$, two-way ANOVA) (Table II). The intensity of staining among the test groups was indistinguishable from that of the myoblast-injected group (Fig. 2C).

At 6 weeks after injection, in the medium-injected group, four samples showed 1 to 8 positive fibers and the other 5 were completely negative. Most of the positive fibers were scattered except in one animal where a cluster containing 8 adjacent positive fibers was found. In MSC-injected muscles, clusters with more than 3 positive fibers were more frequently found. However, the number of the

positive fibers in a cluster was limited to under 8. At 8 weeks, the distribution pattern of the positive fibers was similar to that observed in the 6-week samples. At 10 weeks, there were no dystrophin-positive fibers in 8 of 11 medium-injected muscles. On the other hand, in MSC-injected muscles, 7 muscles showed a cluster containing more than 6 contiguous positive fibers and in one case a marked formation of positive fibers was found.

DISCUSSION

Skeletal muscles develop in a specific sequence of cellular differentiation, which includes: the commitment of progenitor cells into myoblasts, the proliferation of myoblasts, their fusion to form multinucleated myotubes and the sequential expression of muscle-specific proteins. Each of these steps is controlled by a complex spectrum of intrinsic and extrinsic biological factors. Particularly, in the process of myoblast fusion, there is a clear molecular recognition mechanism which allows myoblasts to fuse only to cells of their own lineage and prohibits their fusion to other cell types such as osteogenic, chondrogenic and fibroblastic cells.

In order to account for the spontaneous dystrophin-expression by somatic cell mutation in *mdx* mice with advancing age, the proportion of dystrophin-positive fibers was determined for the MSC-injected right and medium-injected left muscles. Although the incidence of positive fibers was lower in MSC-injected muscles than that of muscles injected with myoblasts, it was significantly higher than that of the controls somatic mutation at each sampling time. Several investigators have proposed that the *mdx* mouse can be used as an animal model to study muscular regenration. It has been shown that myopathic lesions similar to Duchenne-type human muscular dystrophy

are manifest in young *mdx* mice at age 3 to 6 weeks, since no apparent sex-related difference in histology was detected. Injections of reparative cells were done at age 3 weeks into both male and female MDX mice. Our results show that the average rate of conversion was highest at 8 weeks after injection (36%). In comparison, the muscles injected with MSCs showed a cluster of dystrophin-positive myofibers at a significantly lower frequency than the myoblast-injected muscles.

The *in vivo* experiments presented here indicate that MSCs are able to fuse with normal and *mdx* myoblasts and, when injected, can contribute to increased muscle mass, and increased numbers of dystrophin-positive fibers as compared to controls. From these data, taken together, it is reasonable to interpret that some of the increase in muscle mass can be attributed to myoblast- or MSC-induced conversion of dystrophin-negative fibers to dystropin-positive fibers. We postulate that the host provides the signals to cause the MSCs to exhibit a myogenic potential. The result of *in vivo* injectin experiments indicate that implanted bone marrow-derived MSCs differentiate into myogenic cells in regnerating muscles of *mdx* mice. However, the frequency for MSC myogenic conversion is low, so we cannot rule out co-fusion as a possible mechanism for our observations. In summary, MSC's are able to fuse with myotubes and express dystrophin, and are a reservoir which can be utilized for dystrophy-related cell therapies.

We hypothesize that the differentiation efficiency may be increased by the regeneration conditions inherent in *mdx* mice. Therefore, implantation of MSCs in combination with myoblasts or inductive reagents to promote muscle regeneration result in more efficient conversion of

dystrophin-negative to -positive myofibers in the muscle of *mdx* mice.

Example 2

Induction of Myogenisis in Human Volunteers

Bone Marrow Cell Preparation

Bone marrow cells aspirated from the iliac crests of healthy adult human volunteers are used. After soft tissue is completely removed to avoid contamination of myogenic precursors, the marrow is triturated with a syringe filled with Complete Medium and fitted with an 18-gauge needle. The Complete Medium was made up of DMEM-LG (Dulbecco's Modified Eagle's Medium - Low Glucose, Gibco BRL, Grand Island, NY) as more fully described in copending U.S. Serial No. 08/420,297, filed April 11, 1995, the entirety of which is hereby incorporated by reference herein. The Medium contains antibiotics (100 μ/ml sodium penicillin G, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B: Gibco BRL, Grand Island, NY) and is supplemented with 10% fetal calf serum (selected batches). Marrow cells are dispersed several times through 18- and 20-gauge needles. The cells are gently centrifuged, resuspended in Complete Medium, counted and plated at a density of 5.0×10^7 cells/100 mm-dish. At 3 days of culture, the medium is removed, the dishes are washed twice with Tyrode's salts solution (Sigma, St. Louis, MO) and fresh complete medium is added. The medium is changed twice a week.

Periosteal-Derived Cell Preparation

Periosteal cells are obtained from the proximal tibiae of volunteers. The tibial periostea are digested with 0.3% collagenase for 2 hours in a 37°C water bath. At the end of the digestion period, a volume of fetal bovine

serum equal to half the volume of the sample is added. Periosteum-derived cells (periosteal cells) are collected by centrifugation. The pellet of cells and debris are resuspended in 8 ml of Complete Medium and seeded onto 100-mm culture dishes. The plated cells are cultured at 37°C in 95% humidified air plus 5% CO_2, and the medium is changed every 3 days.

Preparation of Myoblasts

Muscle tissue is obtained from muscle biopsies. The dissected muscle is digested with 0.2% trypsin for 35 min. and the reaction is terminated by adding fetal bovine serum at one half the sample volume. The cell suspension is centrifuged for 5 min. at 300 xg, resuspended in 10 ml of DMEM-LG containing 20% fetal calf serum, triturated least 20 times with a 10 ml disposable pipette and then passed through 110 μm Nitex filter (to remove cell aggregates and debris). Before seeding, the cells are preplated in a 100-mm culture dish and incubated for 30 min. to reduce the number of fibroblasts. The nonadherent muscle precursor cells are counted in a hemocytometer and the cell density is adjusted to 1.0×10^5 cells/ml with DMEM-LG supplemented with 20% fetal calf serum. Cells are seeded onto 35 mm plates at 1.0×10^5 cells per plate, in 1 ml, and the medium is changed every 2 days. At the onset of cell fusion (4-5 days), the medium is changed to DMEM-LG with 5% fetal calf serum. The dishes are observed until myotubes form, usually 11 days following initial seeding.

Alternate Preparation of Myoblasts

Myoblasts are also obtained as above. The bone and cartilage are cleared with fine forceps under a dissecting microscope. The dissected muscle tissue is transferred to a sterile tube, 2 ml of Eagle's medium with Earle's salts is added and the tissue is vortexed for 30-60 seconds.

Subsequently, 8-9 ml of Complete Medium [Eagle's Minimum Essential Medium with Earle's salts containing 10% horse serum, 5% embryo extract and antibiotics] are added. The cell suspension is passed through 2 thicknesses of sterile cheesecloth two times and the cells are counted in a hemocytometer. The cell density is adjusted with Complete Medium to 2.0×10^5/ml and one ml is aliquoted onto each 35 mm dish.

Method of Co-culture

A 1 ml of suspension of MPCs is dispersed on gelatin-coated 35-mm culture dishes. Periosteal-derived cells (PCs) labeled with [^{3}H]-thymidine are trypsinized for 5 min. at 37°C with 0.25% trypsin/1 mM ethylenediamine tetra-acetic acid (Gibco BRL, Grand Island, NY), the reaction is terminated by the addition of half volume of calf serum and cells are collected by centrifugation. Subsequently, three different cell suspensions are made at cell densities of 1.0×10^5, 2.0×10^5 and 3.0×10^5 cells/ml separately with Complete Eagle's and Earl's salts Medium. Then, 1 ml of each cell suspension is added to the dishes in which MPCs are seeded previously and mixed with each other. Cells are cultured at 37°C in CO_2 incubator. The medium is changed twice a week.

Activity of Creatine Kinase

Every 2 days after the initiation of the co-culture until day 11, cells are harvested from the dishes by washing twice with cold Tyrode's salt solution twice and stored at -70°C until needed. After thawing, 1 ml of 0.05 M glycylglycine (pH 6.75, Sigma, St. Louis, MO) is added into each dish, and cells are scraped off with a polypropylene policeman, suspended, sonicated for 30 sec. in a Sonifier Cell Disrupter (Model W140D, Branson Sonic Power Co., Plainview, NY) and centrifuged at 12,000 xg for

5 min. The activity of creatine kinase in the supernatant is determined from an assay which couples ATP formation from ADP and creatine phosphate with the formation of DADPH (measured by absorbance at 340 nm), according to Shainberg, *et al.* (34). Briefly, 100 µl of the supernatant are mixed with 890 µl of reaction mixture and the reaction is initiated by adding 10 µl of creatine phosphate (15 mM). The change in adsorbance at 340 nm is measured in a UV160 spectrophotometer (Shimidazu, Tokyo) and the slope of the change in absorbance per min. is used to determine creatine kinase activity.

Autoradiography

First-passage human MSCs and periosteal cells are dispersed into 100-mm dishes at a density of 4.0×10^5 cells in 5 ml of complete medium. After 24 hours, 2.5 µCi of [methyl-^{3}H]thymidine (Amersham, Arlington Heights, IL; 0.5 µCi/ml) are added to each dish. The cells are incubated for 48 hours, washed twice with Tyrode's salts solution and Complete Medium is added to chase unincorporated radioisotope for 48 hours. Subsequently, cells were harvested by digestion with trypsin and counted in a hemocytometer. The cell density is adjusted to 5.0×10^4 cells/ml for MSCs, and 1.0×10^5 cell/ml for periosteum-derived cells. Aliquots of 1.0 ml of each cell suspension are added to the dishes into which twice the number of myoblasts are plated.

At 2-day intervals up to 11 days, co-culture plates are harvested for autoradiography. Randomly chosen dishes are washed twice with cold Tyrode's salts solution, and the cells are fixed with 10% neutral formalin. After the cells are dehydrated in graded ethanol and air-dried, the dishes are coated with NTB-2 autoradiography emulsion (Eastman Kodak, Rochester, NY) and stored in the dark at -20°C for 7

days. Subsequently, the dishes are developed and air-dried, and the cells are observed under a microscope (BH-2, Olympus, Tokyo).

Autoradiography with Immunohistochemistry

Co-cultures of MSCs with myoblasts are immunostained with anti-dystrophin antibody prior to autoradiography. At 9 days, the dishes are washed twice with Tyrode's salts solution and the cells are fixed with cold methanol for 15 min. After washing with Tyrode's salts solution, PBS+HS (phosphate buffered saline pH 7.4, containing 10% horse serum; JRH Biosciences, Lenesa, KS) is added onto the cells. The cells are incubated for 2.5 hours in a humid chamber with anti-dystrophin antibody diluted to 1 to 250 with PBS+HS. After incubation, the cells are washed 4 times with PBS+HS for 5 min. each and incubated with biotinylated sheep anti-sheep IgG (Vector Laboratories, Inc., Burlingame, CA) and diluted (1:500) in PBS +HS. After washing 4 times, the cells are exposed to streptavidin-horseradish peroxidase conjugate (Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD) for 30 min. To develop color, the cells are immersed in the solution of diaminobenzidine (DAB) (0.2 mg/ml) in 20 mM (tris)hydroxyethyl aminoethane, 150 mM sodium chloride, pH 7.6 containing hydrogen peroxide (0.01%) at room temperature for 15 min. in the dark. The cells are washed once with PBS and twice with distilled water and then exposed to 10% copper nitrate for 1 min. to intensify the DAB product. After washing again with distilled water, cells are then dehydrated in graded ethanol and air-dried, and autoradiography is performed as described above.

The invention has been described and illustrated with reference to the preferred embodiment. Modifications and

alterations will occur to others upon reading and understanding the preceding detailed description. It is intended that the invention be construed as including all such modifications and alterations insofar as they come within the scope of the appended claims or the equivalents thereof.

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What Is Claimed Is:

1. A composition of matter which comprises a mixture of isolated human muscle precursor cells and isolated human mesenchymal stem cells.

2. The composition of claim 1 wherein the mesenchymal stem cells are marrow-derived.

3. The composition of claim 1 wherein the mesenchymal stem cells are periosteum-derived.

4. The composition of claim 1 which further comprises complete medium.

5. The composition of claim 1 which further comprises a myoinductive agent.

6. The composition of claim 5 wherein the myoinductive agent is selected from the group consisting of 5-azacytidine and 5-azadeoxycytidine.

7. A composition of matter which comprises a mixture of isolated muscle precursor cells and isolated periosteum cells.

8. The composition of claim 7 wherein there are at least as many muscle precursor cells in the mixture as there are periosteum cells.

9. The composition of claim 7 which further comprises a myoinductive agent.

10. The composition of claim 7 wherein the myoinductive agent is selected from the group consisting of 5-azacytidine and 5-azadeoxycytidine.

11. A method for inducing isolated human mesenchymal stem cells to differentiate into myogenic cells which comprises maintaining said cells in the presence of muscle precursor cells.

12. The method of claim 11 wherein the isolated human mesenchymal stem cells are maintained in the presence of muscle precursor cells *in vitro*.

13. The method of claim 11 wherein the isolated human mesenchymal stem cells are maintained in the presence of muscle precursor cells *in vivo*.

14. A method for producing dystrophin-positive myogenic cells in a mammal which comprises administering to the mammal a myogenic cell producing amount of isolated mesenchymal stem cells.

15. A method for producing dystrophin-positive myogenic cells in a mammal which comprises administering to the mammal a myogenic cell producing amount of the composition of claim 1.

16. A method for producing dystrophin-positive myogenic cells in a mammal which comprises administering to the mammal a myogenic cell producing amount of the composition of claim 5.

17. A method for producing dystrophin-positive myogenic cells in a mammal which comprises administering to the mammal a myogenic cell producing amount of the composition of claim 7.

18. A method for effecting muscle regeneration in an individual in need thereof which comprises administering to

a individual in need of muscle regeneration a muscle regenerative amount of isolated mesenchymal stem cells.

19. A method for effecting muscle regeneration in an individual in need thereof which comprises administering to a individual in need of muscle regeneration a muscle regenerative amount of the composition of claim 1.

20. A method for effecting muscle regeneration in an individual in need thereof which comprises administering to a individual in need of muscle regeneration a muscle regenerative amount of the composition of claim 5.

21. A method for effecting muscle regeneration in an individual in need thereof which comprises administering to a individual in need of muscle regeneration a muscle regenerative amount of the composition of claim 7.

22. A method for treating muscular dystrophy in an individual so afflicted which comprises treating muscular dystrophy in an individual having muscular dystrophy by administering to that individual an amount of isolated mesenchymal stem cells effective to produce dystrophin-positive myogenic cells in said individual.

23. A method for treating muscular dystrophy in an individual so afflicted which comprises treating muscular dystrophy in an individual having muscular dystrophy by administering to that individual an amount of the composition of claim 1 effective to produce dystrophin-positive myogenic cells in said individual.

24. A method for treating muscular dystrophy in an individual so afflicted which comprises treating muscular dystrophy in an individual having muscular dystrophy by

administering to that individual an amount of the composition of claim 5 effective to produce dystrophin-positive myogenic cells in said individual.

25. A method for treating muscular dystrophy in an individual so afflicted which comprises treating muscular dystrophy in an individual having muscular dystrophy by administering to that individual an amount of the composition of claim 7 effective to produce dystrophin-positive myogenic cells in said individual.

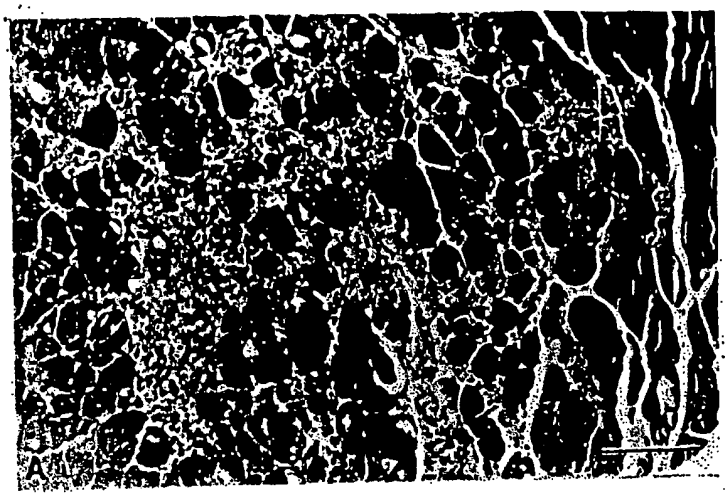

FIG. 1A

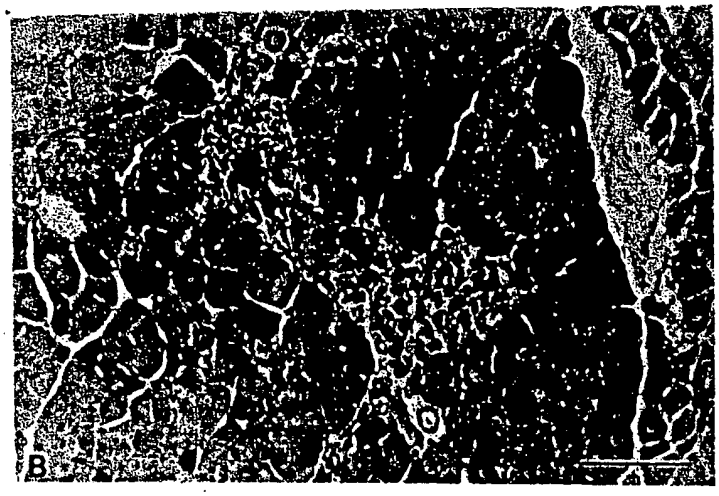

FIG. 1B

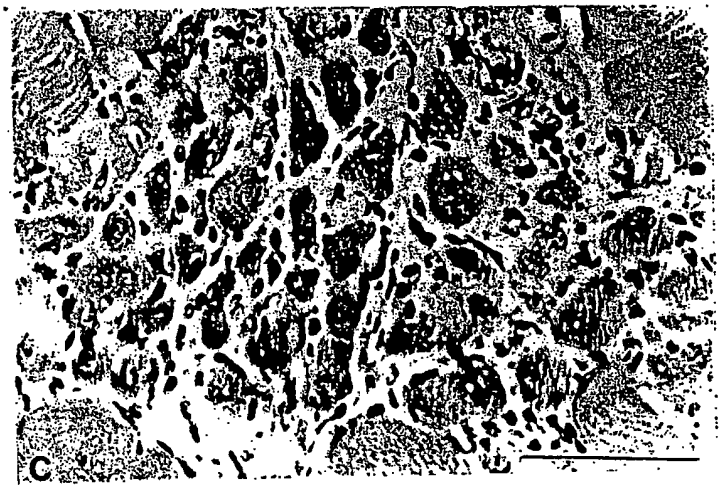

FIG. 1C

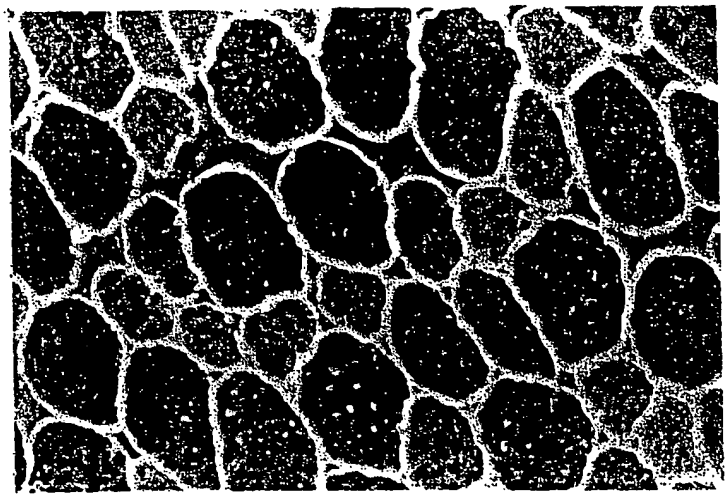

FIG. 2A

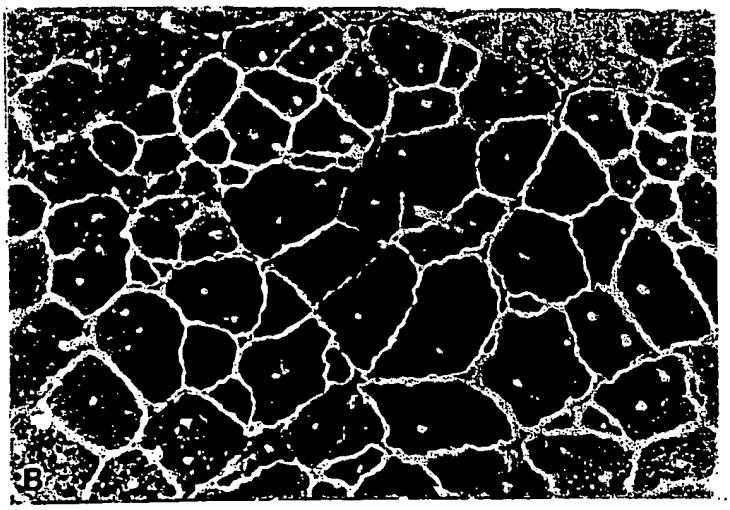

FIG. 2B

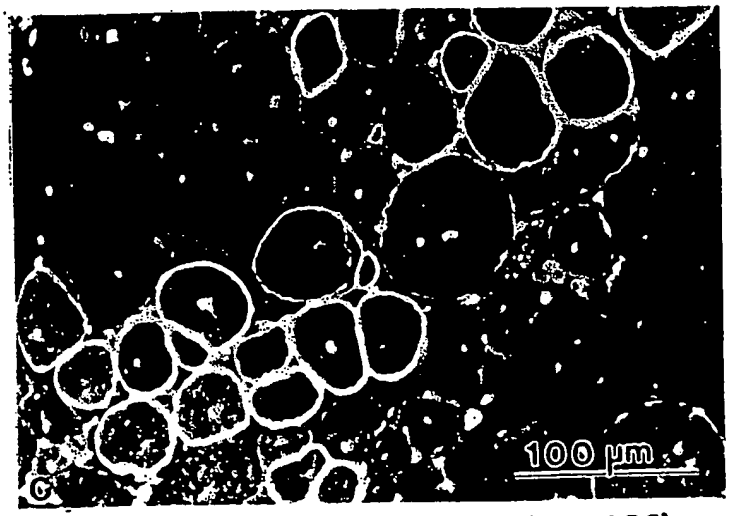

FIG. 2C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08722

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A01N 63/00, 65/00; A61K 35/34, 35/26, 35/28; C12N 5/00
US CL :424/93.3, 93.7, 548, 577; 435/240.2; 514/907
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/93.3, 93.7, 548, 577; 435/240.2; 514/907

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE, CA, EMBASE, BIOSIS, INPADOC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	MORI, N. et al. Ultrastructural Findings in the Wound Healing of the Colonic Mucosa of Rabbits. Acta Anat. January 1989, Vol. 134, No. 1, pages 82-88, especially pages 82 and 83.	1 --- 2, 3, 4, 12, 13
Y	CAPLAN, A.I. The Mesengenic Process. Clinics in Plastic Surgery. July 1994, Vol. 21, No. 3, pages 429-435, especially pages 429 and 430.	2, 3
X, P	WAKITANI, S. et al. Myogenic Cells Derived from Rat Bone Marrow Mesenchymal Stem Cells Exposed to 5-Azacytidine. Muscle & Nerve. December 1995, Vol. 18, No. 12, pages 1417-1426, see entire article.	1-6, 11-13, 15, 16, 19, 20, 23, 24.

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
27 AUGUST 1996

Date of mailing of the international search report
13 SEP 1996

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08722

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 5,435,999 A (AUSTIN) 25 July 1995 (25.07.95), see entire patent.	15, 16, 19, 20, 23, 24
A, P	US 5,486,359 A (CAPLAN ET AL.) 23 January 1996 (23.01.96).	1-6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08722

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6, 11-13, 15, 16, 19, 20, 23, 24

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08722

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-6, 11-13, 15, 16, 19, 20, 23, and 24, drawn to a composition of matter, a method for inducing isolated human mesenchymal stem cells to differentiate into myogenic cells, methods for producing dystrophin-positive myogenic cells in a mammal, methods for effecting muscle regeneration in an individual in need thereof, and methods for treating muscular dystrophy in an individual so afflicted.

Group II, claims 7-10 drawn to a composition of matter.

Group III, claim 17 drawn to a method for producing dystrophin-positive myogenic cells in a mammal.

Group IV, claim 21 drawn to a method for effecting muscle regeneration in an individual in need thereof.

Group V, claim 25 drawn to a method for treating muscular dystrophy in an individual so afflicted.

Group VI, claim 14 drawn to a method for producing dystrophin-positive myogenic cells in a mammal.

Group VII, claim 18 drawn to a method for effecting muscle regeneration in an individual in need thereof.

Group VIII, claim 22 drawn to a method for treating muscular dystrophy in an individual so afflicted.

The inventions listed as Groups I-VIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: for example, the inventions of Group I all require human muscle precursor cells and isolated human mesenchymal stem cells. The inventions of Group II require isolated muscle precursor cells and isolated periosteum cells. The inventions of Group III just require administration of isolated mesenchymal stem cells to produce dystrophin-positive myogenic cells, and the invention of Group IV requires a muscle regenerative amount of isolated mesenchymal stem cells. Groups V-VIII are directed to methods which are independent of one another and do not have to be practiced together.

The eight groups are not linked by a special technical feature since each requires a different primary ingredient not required to practice the inventions of the other groups. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.